

Sexual Differentiation in *Ligustrum micranthum* (Oleaceae), Endemic to the Bonin (Ogasawara) Islands

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Ligustrum micranthum Zucc. (Oleaceae), a small tree endemic to the Bonin Islands, was examined in the field to clarify its sexual system. In the local populations examined on Chichi-jima, all individuals appeared to be hermaphroditic, having a pistil with an ovary and two stamens. They were divided into two morphological groups, however, one having anthers with pollen (staminate fertile) and the other having anthers without pollen (staminate sterile). Staminate sterile individuals set fruit under open pollination. However, staminate fertile individuals showed various fruit production under open pollination. Five plants of the 19 staminate fertile individuals examined did not set fruit for the two years of our observations. This is the first report of sexual polymorphism in *Ligustrum*. Such a sexual system in *L. micranthum* might have evolved within the Bonin Islands.

Key words: Bonin (Ogasawara) Islands, *Ligustrum micranthum*, gynodioecy, oceanic islands, Oleaceae, pollen stainability, sexual differentiation, subdioecy

Flowering plants display a wide variety of sexual systems, such as hermaphroditism, dioecy, gynodioecy, androdioecy and so on (Darwin 1877, Bawa & Beach 1981, Richards 1997). Various sexual systems have risen independently in many flowering plant families throughout the world (Sakai & Weller 1999), but the sexual system of many species of plants remain unclear and occasionally have been wrongly described.

Ligustrum L. (Oleaceae) comprises about 40 species and is distributed mainly from Europe to eastern Asia, New Guinea, and Australia (Mabberley 2008). *Ligustrum micranthum* Siebold et Zucc., among 10 species that have been recorded from Japan (Noshiro 1993), is endemic to the Bonin (Ogasawara) Islands and has been regarded to be hermaphroditic (Abe 2006). Our preliminary observations suggested that some sexual differentiation was taking place in *L. micranthum*.

The Bonin Islands form an oceanic island group that has never been connected to a land-mass during its geological history (Asami 1970). On such oceanic islands, many endemic plant species are present (Carlquist 1974), and they often show sexual systems, such as dioecy, that promote outcrossing (Sakai *et al.* 1995). Previous studies in the Bonin Islands have reported several dioecious species of plants that could have evolved from hermaphroditic ancestors (*Calli-carpa*, Kawakubo 1990; *Dendrocacalia*, Kato & Nagamasu 1995; *Wikstroemia*, Sugawara *et al.* 2004). Additionally, androdioecy, a rare sexual system, was recently reported for *Morinda umbellata* subsp. *boninensis*, a Bonin Island endemic (Nishide *et al.* 2009). Many endemic plant species on islands exhibit a different sexual system from their relatives on the main islands of Japan and/or from other nearby areas. In the present

study, we examined floral differentiation, pollen stainability, and reproduction in *L. micranthum* to determine its sexual system.

Materials and methods

Ligustrum micranthum is a small, evergreen tree endemic to the Bonin Islands, usually in scrublands on rocky ridges in coastal areas, and sometimes on the forest floor in mountainous areas (Shimizu 1992). It flowers from early April to late May. In the present study, we examined 49 individuals at two sites, Asahi-yama and Tatsumi on Chichi-jima in the Bonin Islands, Tokyo Prefecture.

To determine sexual differentiation in the flowers, one to two inflorescences were collected from each plant and preserved in 70% ethanol. The sexual mode of each plant was determined by examining for the presence or absence of pollen and the production of fruits and seeds. Pollen stainability (fertility) using aniline blue in lactophenol of more than 200 grains per individual was also determined. To evaluate pollen stainability, we examined two flowers per plant. To confirm the presence of ovules within each ovary, we examined 10 flowers using methods described in the following paragraph. We also measured corolla lobe length, filament height, anther length, anther width, stigma height, and stigma size

(Fig.1). Measurements were done on one to three flowers per plant using a digital microscope (VHX-600, KEYENCE). In total, 44 out of 49 individuals were examined. The data obtained from all the individuals examined were subjected to principal component analysis (PCA) (Jump ver.5, SAS Institute Inc). Voucher specimens were deposited in the Makino Herbarium (MAK) of Tokyo Metropolitan University.

Stigmatic papillae and ovules within each ovary were observed using scanning electron microscopy (SEM). Two flower samples, each from two different forms of plant as described below, were collected at the Asahi-yama and Tatsumi sites. The four samples were dehydrated in an ethanol:t-butanol series, freeze-dried using a freeze drying device (JFD-300, JEOL), mounted onto SEM stubs using double-sided carbon tape, coated with gold using an ion sputter (JFC-110E, JEOL) and observed using a scanning electron microscope (JSM-5600LV, JEOL).

To determine fruiting and percent fruit set per inflorescence, 1 to 3 inflorescences from 35 of the 49 marked plants were examined. To avoid overlooking fruiting in the 35 plants, we also examined additional inflorescences (except marked ones) on June 16, 2009, and July 7, 2010, to detect the presence of fruit over the two fruiting seasons. We also examined fruit production in each group. We counted the number of flowers of 1 to 3 inflorescences from 37 individuals at the Asahi-yama and Tatsumi sites.

To compare fruit productivity between the two groups, we counted the number of fruits in each inflorescence. Inflorescences that did not set fruit were excluded from our analysis. For the results, six sterile staminate individuals, four from Asahi-yama and two from Tatsumi, as well as three fertile staminate individuals, two from Asahi-yama and one from Tatsumi, were used for the analysis. We compared the percentage of fruit productivity between the two groups. The number of individuals examined was limited, because fruit production was low at both sites.

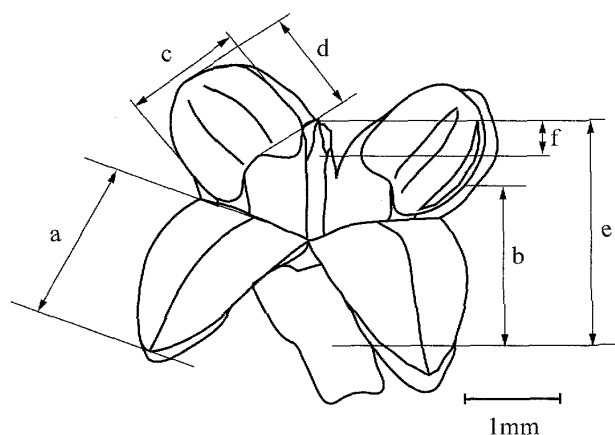


FIG. 1. Measurements of flowers. a, corolla lobe length; b, filament height; c, anther width; d, anther length; e, stigma height; f, stigma size.

Results

Pollen productivity and floral morphology

The flowers of *Ligustrum micranthum* are white, symmetrical, with a short corolla tube, two stamens and a superior ovary with four ovules. The anthers and a style mostly protruded from the corolla tube. The filaments are adnate to the corolla tube at their base.

Pollen stainability revealed natural populations of *Ligustrum micranthum* to comprise two types of individuals differing in pollen productivity: staminate sterile and staminate fertile individuals (Fig. 2). The staminate sterile individuals completely lacked pollen, while the staminate fertile individuals produced a large amount of pollen, with stainability mostly above 90% at both study sites (Fig. 3).

The two kinds of individuals also showed differences in floral morphology (Fig. 2). Data from the six floral characters were subjected to principal component analysis (PCA). Two-dimensional diagrams based on the first three components obtained by PCA are shown in Fig. 4. The first three principal components explain 85.1% of the total

variance (Table 1). Corolla lobe length, filament height, anther length and stigma height mainly contributed to the first components (PC-1); anther width and stigma length contributed to the second component (PC-2); and anther width largely contributed to the third component (PC-3). As shown in Fig. 4, individual plants were placed into two groups: those with and those without pollen. Statistically, anther width and anther length in the staminate fertile group were larger than in the staminate sterile group (Wilcoxon test, $p < 0.05$; Table 2). In the Asahi-yama site, stigma height and stigma size of the staminate sterile individuals were also significantly greater than in the staminate fertile individuals (Wilcoxon test, $p < 0.01$). At the Tatsumi site, however, stigma height and stigma size of the staminate sterile individuals tended to be greater than in the staminate fertile individuals, but the difference was not significant. The difference in corolla lobe length between the two groups was insignificant at both sites (Table 2).

Observation on floral morphology related to carpellate function

Scanning electron micrographs of the stigma

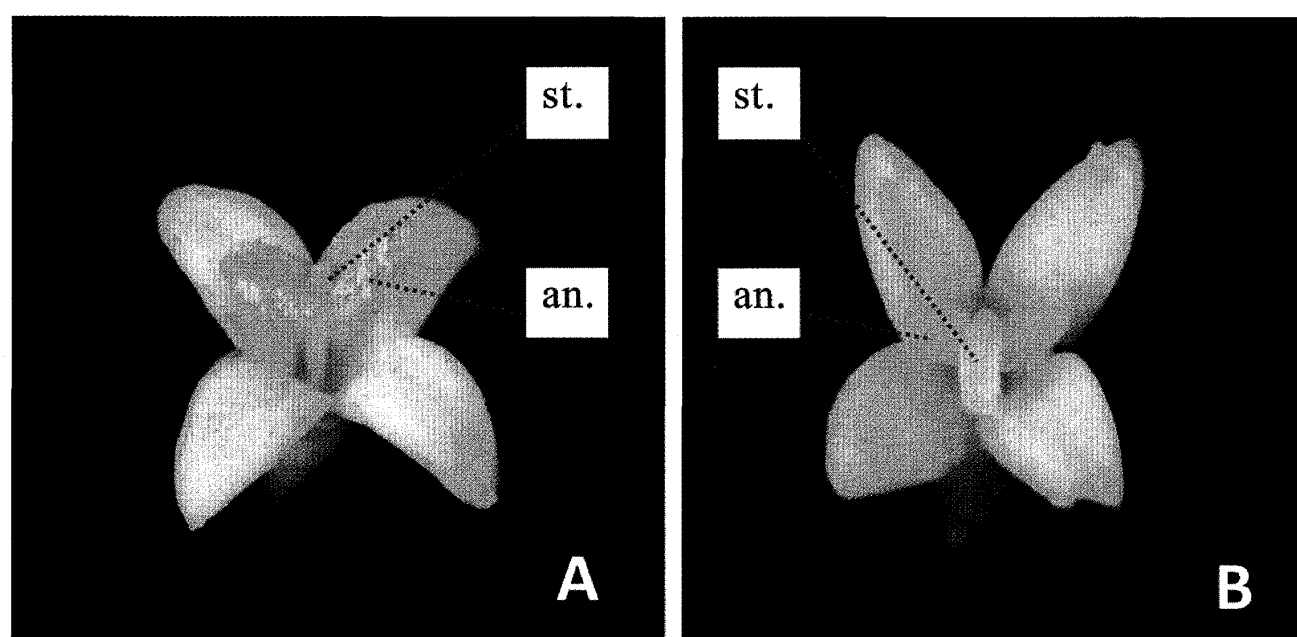


FIG. 2. Flowers with pollen (A) and staminate sterile flowers (B) of *Ligustrum micranthum* from the Asahi-yama site on Chichijima. an, anther; st, stigma.

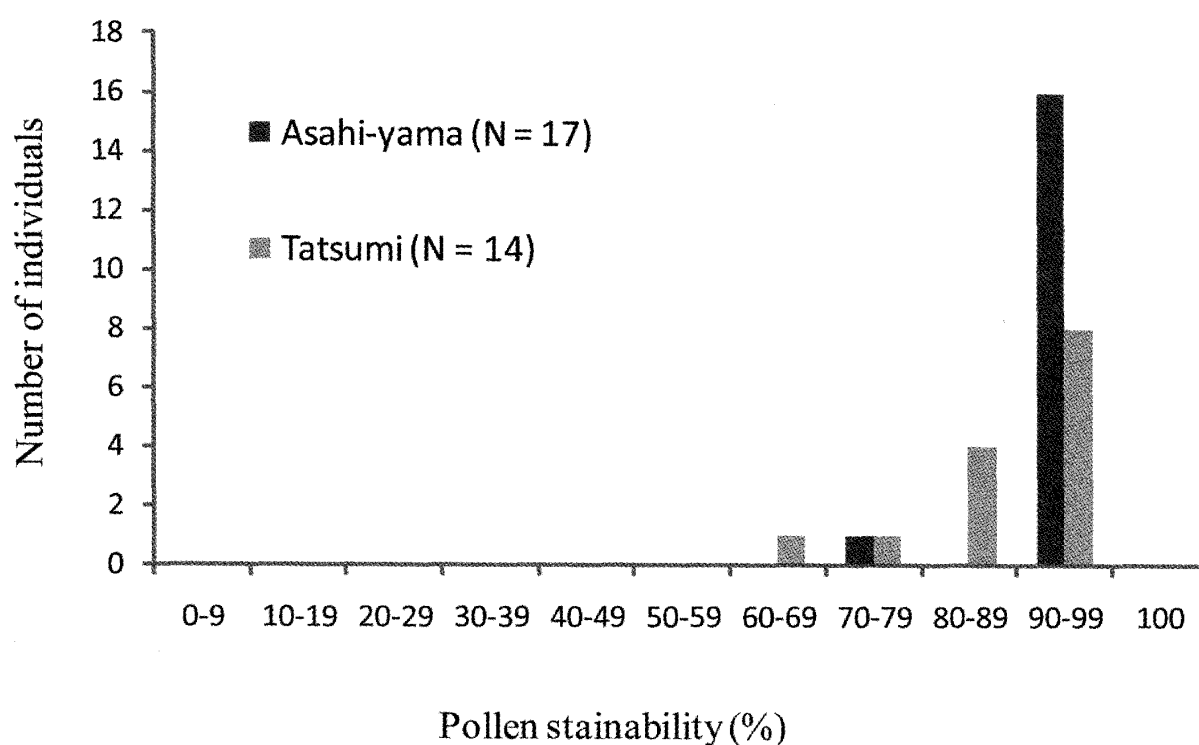


FIG. 3. Pollen stainability in staminate fertile individuals of *Ligustrum micranthum* on Asahi-yama and at Tatsumi on Chichijima. Values are based on means from two flowers per individual. N: number of individuals examined.

TABLE 1. Factor loading, eigenvector, cumulative variance for principal component analysis in 44 individuals of *Ligustrum micranthum*.

Principal component No.	1	2	3
Cumulative variance (%)	46.7	76.4	85.1
Characters	Eigenvectors		
Corolla lobe length	0.51	-0.16	0.19
Filament height	0.44	0.29	-0.33
Anther width	0.24	0.54	0.77
Anther length	0.41	0.38	-0.48
Stigma height	0.49	-0.33	0.03
Stigma length	0.29	-0.58	0.17

and transverse sections of the ovaries of the two plant groups are shown in Fig. 5. We could not find major differences between the two groups in the development of stigmatic papillae and ovules. The gynoecia of the two groups appeared to be functional, at least as observed from the morphology.

Fruit production under open pollination

Results from observations of fruiting in all the individuals at the two study sites over two flowering seasons are summarized in Table 3. At both sites, the staminate sterile individuals (16 plants) showed good fruit set; 15 plants set fruit in both seasons, and 1 plant set fruit only in 2010. Five plants of the 19 staminate fertile individuals

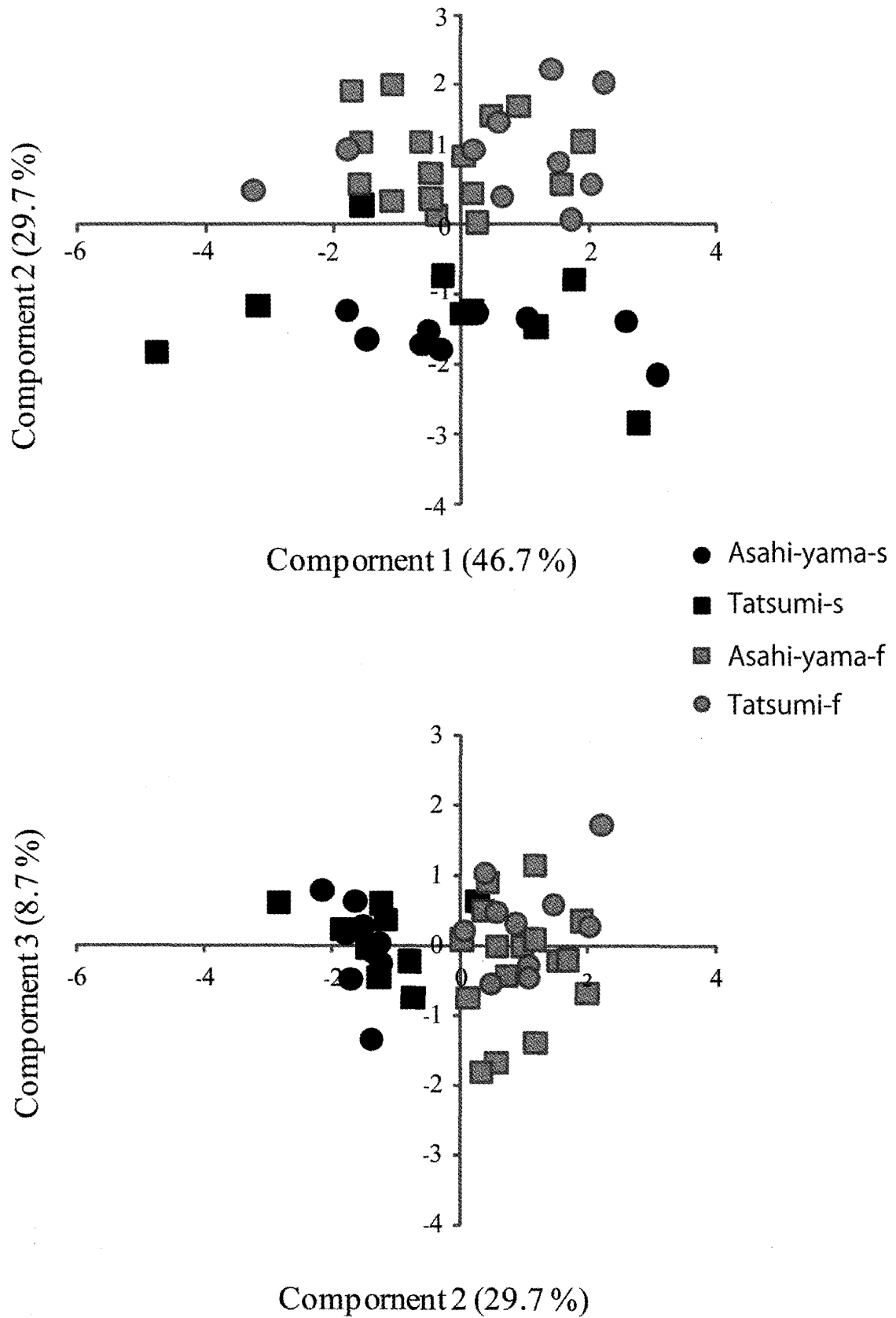


FIG. 4. Two dimensional diagram of 44 individuals of *Ligustrum micranthum* from two sites on Chichi-jima based on principal component analysis of morphological data from flowers. f: staminate fertile individuals. s: staminate sterile individuals.

TABLE 2. Means values and standard deviations (SD) of six morphological characters in staminate fertile and staminate sterile individuals at Asahi-yama and Tatsumi sites on Chichi-jima. Difference between two morphs statistically examined using Wilcoxon test. N: number of individuals examined.

Site	Pollen present			Pollen absent			Wilcoxon-test
Character	N	Mean \pm S.D.	Range	N	Mean \pm S.D.	Range	
Asahiyama							
Corolla lobe length (mm)	16	1.60 \pm 0.18	1.31–1.90	9	1.74 \pm 0.30	1.44–2.37	ns
Filament height (mm)	16	2.22 \pm 0.30	1.73–2.76	9	2.00 \pm 0.33	1.56–2.29	ns
Anther width (mm)	16	0.74 \pm 0.10	0.51–0.99	9	0.60 \pm 0.07	0.51–0.72	**
Anther length (mm)	16	1.19 \pm 0.14	0.94–1.48	9	1.05 \pm 0.15	0.84–1.19	*
Stigma height (mm)	16	2.43 \pm 0.23	1.81–2.86	9	2.79 \pm 0.25	2.54–3.20	**
Stigma length (mm)	16	0.49 \pm 0.09	0.39–0.77	9	0.74 \pm 0.09	0.65–0.90	**
Tatsumi							
Corolla lobe length (mm)	10	0.94 \pm 1.48	1.19–1.93	9	1.66 \pm 0.33	1.22–2.16	ns
Filament height (mm)	10	2.37 \pm 0.33	1.30–2.57	9	1.90 \pm 0.36	1.30–2.34	ns
Anther width (mm)	10	0.85 \pm 0.15	0.41–0.75	9	0.61 \pm 0.10	0.41–0.75	**
Anther length (mm)	10	1.18 \pm 0.14	0.52–2.57	9	0.98 \pm 0.22	0.52–1.17	*
Stigma height (mm)	10	2.62 \pm 0.37	2.06–3.53	9	2.78 \pm 0.48	2.06–3.53	ns
Stigma length (mm)	10	0.54 \pm 0.12	0.47–0.90	9	0.63 \pm 0.13	0.47–0.68	ns

**: $p < 0.01$, *: $p < 0.05$, ns; not significant.

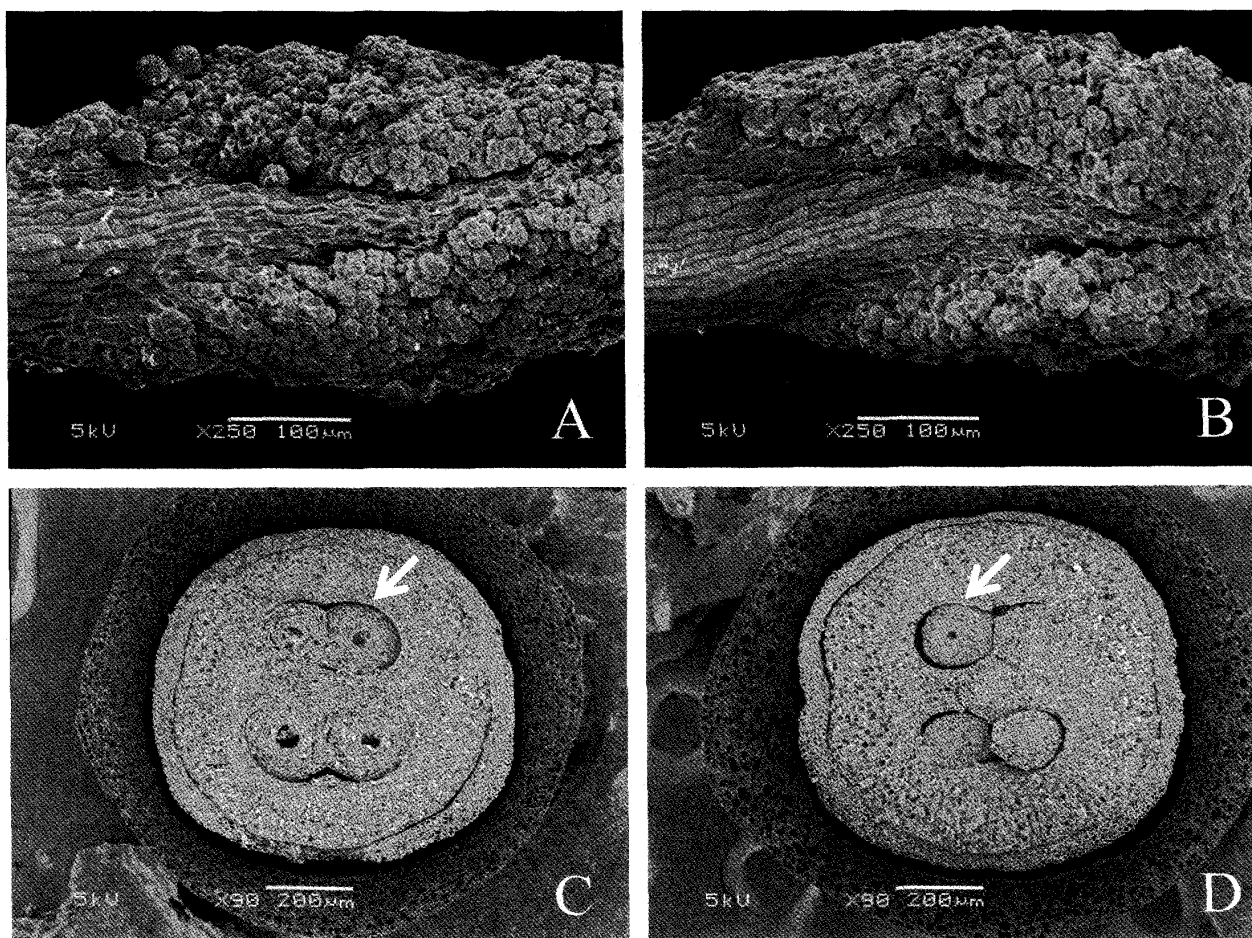


FIG. 5. SEM photographs of stigmas (A, B) and longitudinal sections of ovaries (C, D) in staminate fertile and staminate sterile individuals of *Ligustrum micranthum*. A, C: flower of staminate fertile individual. B, D: flower of staminate sterile individual. Arrows indicate ovules within each ovary.

examined did not set fruit during the two seasons, and 8 plants set fruit in only one season. Only 6 of the 19 staminate fertile individuals set fruit in both seasons.

The mean fruit set percentage per inflorescence was $18.76 \pm 13.51\%$ for staminate sterile individuals ($n = 6$), and $12.37 \pm 13.66\%$ for staminate fertile individuals ($n = 4$). Percent fruit set per inflorescence did not differ significantly between the two groups (Wilcoxon test, $p = 0.252$).

Discussion

Sexual system in Ligustrum micranthum

As described above, *Ligustrum micranthum* in the Bonin Islands includes individuals with and without pollen in their anthers. The staminate fertile group has significantly larger anthers than the staminate sterile group (Table 2). The flowers of individuals producing pollen also an ovary and stigma. Staminate sterile individuals lack pollen in their anthers, but the flowers of those plants have an ovary and stigma. Morphologically, staminate fertile individuals are therefore hermaphrodites, whereas staminate sterile individuals are carpellate.

Fruit production in the two groups also differs. All staminate sterile individuals, with the exception of one, set fruit in both years of our observations. In contrast, fruit production in the staminate fertile individuals, which are morphologically hermaphroditic, was variable. More than half of the staminate fertile individuals did not set fruit during either one or two fruiting seasons. Five out of the 19 staminate fertile individuals did not set fruit during the two years of our observations.

Staminate fertile and staminate sterile individuals show clear differences in fruit production. Ovule production in the staminate sterile group appears greater than in the staminate fertile group. Although both groups are functionally carpellate—they both bear ovaries and a stigma—they may differ in fruit production.

To confirm difference in fruit production between the two groups, further detailed investigations are needed, since there are other possible

TABLE 3. Fruit set in individuals observed over two seasons (2009 and 2010) at the two study sites, Asahi-yama and Tatsumi on Chichi-jima.

Sample No.	pollen grains	Fruitage	
		2009	2010
Asahi yama 1	a	+	+
Asahi yama 2	a	+	+
Asahi yama 7	a	+	+
Asahi yama 9	a	+	+
Asahi yama 11	a	+	+
Asahi yama 14	a	+	+
Asahi yama 17	a	+	+
Asahi yama 18	a	+	+
Asahi yama 4	p	+	—
Asahi yama 5	p	+	+
Asahi yama 6	p	+	+
Asahi yama 8	p	—	+
Asahi yama 10	p	+	+
Asahi yama 13	p	+	—
Asahi yama 15	p	+	+
Asahi yama 16	p	—	—
Asahi yama 21	p	—	+
Asahi yama 22	p	—	—
Asahi yama 23	p	—	+
Tatsumi 1	a	+	+
Tatsumi 2	a	+	+
Tatsumi 7	a	—	+
Tatsumi 9	a	+	+
Tatsumi 11	a	+	+
Tatsumi 12	a	+	+
Tatsumi 13	a	+	+
Tatsumi 20	a	+	+
Tatsumi 4	p	—	—
Tatsumi 5	p	—	—
Tatsumi 6	p	—	—
Tatsumi 10	p	—	+
Tatsumi 14	p	—	+
Tatsumi 15	p	+	+
Tatsumi 16	p	+	+
Tatsumi 19	p	—	+

p, staminate fertile individuals; a, staminate sterile individuals. +: fruit formed. —: fruit not formed.

causes for lower fruit production in staminate fertile (morphologically hermaphroditic) individuals.

One possible explanation for reduced fruit production in some endemic species in the Bonin Islands may be pollinator limitation (Watanabe 2008). When pollinators are limited, fruit production in self-incompatible individuals is reduced more than in self-compatible individuals (Larson & Barrett 2000). Since carpellate individuals do not produce pollen, and morphologically hermaphroditic individuals do, fruit production is presumed to be reduced. Fruit production in staminate sterile individuals (carpellate plants), however, was higher than in staminate fertile individuals (morphological hermaphrodites) according to our data. Pollinator limitation therefore does not appear to be the main factor for decreased fruit production in staminate fertile individuals. Pollinator limitation, though, can result in reduced fruit production in hermaphrodites if they are self-incompatible or suffering from severe inbreeding depression. Further studies of the staminate fertile individuals and their reproductive traits as well as observations of pollinator behavior to confirm if pollinator limitation indeed causes a reduction in fruit production in staminate fertile (morphologically hermaphroditic) individuals of *Ligustrum micranthum* are needed.

If there is inbreeding depression, fruit production in hermaphroditic individuals can be reduced by geitonogamy (Harder & Barrett 1995, Barrett 2002, Finer & Morgan 2003). When self-incompatibility exists, stigmas clogged by self-pollination may result in considerable reduction in fruit production in hermaphrodites (Kikuzawa 1989, Broyles and Wyatt 1993). Hand-pollination experiments and observations of the stigma of both staminate fertile (morphologically hermaphroditic) individuals and staminate sterile (carpellate) individuals are needed to determine whether reduction in fruit production is the result of clogging or geitonogamy.

Another possible explanation for reduction in fertility is that the staminate fertile individuals may be differentiated into two types; one with

and one without carpellate function. The staminate fertile individuals that set little fruit may be functionally staminate, while their carpellate function is weaker. If true, carpellate, staminate and hermaphroditic individuals would comprise the populations of *Ligustrum micranthum*. Such a sexual system, called subdioecy, consists of a population of plants with staminate flowers, pistillate flowers, and some hermaphrodites (Sakai and Weller 1999). From an evolutionally point of view, subdioecy has been regarded as an intermediate stage between gynodioecy and dioecy (Bailey & Delph 2007, Barrett 2010). Examples of subdioecious systems have been reported in *Hebe subalpina* (Plantaginaceae) (Delph 1990) and *Ochradenus baccatus* (Resedaceae) (Wolfe & Shmida 1997). To determine if *Ligustrum micranthum* exhibits a subdioecious sexual system, it is important to know if the pollen is functional while the ovaries and ovules are not. Hand-pollination experiments and more detailed observations of fruit and seed production are necessary.

As described above, *Ligustrum micranthum* in the Bonin Islands is sexually polymorphic. It has at least two different sexual morphs; carpellate and morphologically hermaphroditic. In *Ligustrum*, all species have so far been regarded as being sexually hermaphroditic (Chang *et al.* 1996). *Ligustrum micranthum* is the only species in the genus that shows sexual polymorphism. Although further examinations are needed to determine sexual function in morphological hermaphrodites, it is possible that the sexual system of *Ligustrum micranthum* evolved within the Bonin Islands from a hermaphroditic ancestor.

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